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Serologically Based Diagnostic and Quantification Tests for Nematodes*

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Abstract: Plant parasitic nematodes are important plant pests which are difficult to identify and quantify. Serologically based techniques offer new ways to identify and quantify these parasites. Several factors need to be considered when developing an immunoassay for plant parasitic nematodes, including the application of the assay and the extraction procedure which together determine the likely organisms with which antibodies may cross-react. The four major factors: taxon specificity, nematode stage specificity, antibody specificity, and the level of management which is possible, need to be addressed interdependently and the best possible compromise made to develop a practical assay. Progress has been made in the immuno-diagnosis and quantification of potato cyst nematodes (*Globodera* spp) and root-knot nematodes (*Meloidogyne* spp) and these two groups of nematodes present different problems and therefore different challenges. The research reported here compares and contrasts work on these two groups of nematodes and highlights some of the important factors to be considered in the development of immunoassays for plant parasitic nematodes.

Key words: ELISA, *Globodera*, identification, *Meloidogyne*, monoclonal antibodies.

1 INTRODUCTION

The widespread concern about the health and environmental hazards of pesticides has led to considerable international efforts directed towards reduction of their use.[1,2] In this regard nematicides are no exception.[3] The biomanagement of pests relies upon biological diversity, either in the form of genetic resistance to the pests or hyperparasites, as the basis of developing a sustainable agricultural system. Underpinning this management strategy, up-to-date qualitative and quantitative data on pest populations are required. Plant parasitic nematodes are microscopic animals with a highly conserved morphology, and are, therefore, notoriously difficult to identify even by highly trained personnel. New management strategies will require simple, rapid and reliable methods for the identification and quantification of nematode pests which will enable the routine monitoring of nematode populations. The advent of molecular biology has brought with it a number of new techniques for analysing DNA, protein, carbohydrates and lipids which can be helpful in the identification of pests; of these, allozyme-, monoclonal antibody- and DNA-based systems are the most well developed for nematodes and have been extensively reviewed.[4–10] Each technique has its strengths and weaknesses and the authors refer readers to these comprehensive reviews for comparisons of the different methods. Qualitative diagnostic tests which require the detection of nematodes present in small numbers are essential for quarantine and certification schemes, but one of the most intractable problems in nematology is the development for use by agricultural advisers of routine quantitative assays which are able to combine a high degree of both specificity and sensitivity. Quantitative assays using molecular techniques have to rely on diagnostic probes and as the technology for enzyme-linked immunoabsorbent assays (ELISA) is more highly developed than for quantitative DNA-based assays,[11] the present paper will concentrate on ELISA. To date, most research has been aimed at developing assays for root-knot (*Meloidogyne*

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spp) and potato cyst (*Globodera* spp) nematodes. It is the purpose of this paper to highlight the considerations which must be taken into account in the development of a quantitative immunoassay for these nematodes, with especial reference to research at IACR-Rothamsted.

2 GENERAL CONSIDERATION FOR THE DEVELOPMENT OF AN IMMUNOASSAY

A number of practical and theoretical considerations are necessary before a quantitative immunoassay is developed (Fig. 1). One of the first questions to ask is simply whether or not an immunologically based assay would represent an improvement over presently available techniques; immunoassays are costly to develop and if the nematode problem, e.g. the presence of cysts on roots, can be assessed, for example, by eye with the help of a magnifying glass, then this is preferable to the development of a sophisticated assay. Once it has been established that an assay is required, its stringency will be determined by how widely it is envisaged the assay will be used. One developed for a crop grown in a particular locality, e.g. potatoes within the UK, and with few other nematodes with which the pest can be confused, will be much less rigorous than if the assay is to be used on a world-wide basis. Having decided the geographical scale on which the assay has to be used, it is necessary to identify the needs of the end-users at an early stage; a laboratory undertaking quarantine and plant health certification will have very different requirements from an agricultural extension or advisory service. Similarly, assays developed as research tools for screening nematode-resistant germplasm or for nematicide screening trials will have requirements very different from those of the two users mentioned above. An important consideration is also the type of matrix (soil, water, roots etc.) in which the nematode assay will be performed; this is related to the extraction and antigen release procedures which will be undertaken prior to performing the assay. Having decided on the geographical locality and the stringency with which the assay has to perform, a number of criteria have to be assessed concerning the specificity of the immunoassay. It is here that decisions have to be made regarding the requirements of the type and specificity of the antibody(ies) to be used. The level of specificity required will depend on the level of discrimination required for the purposes of the assay. Four factors will need to be addressed interdependently and the best possible compromise made. These are taxon specificity, nematode stage specificity, antibody specificity and the type of management which is possible (Fig. 1).

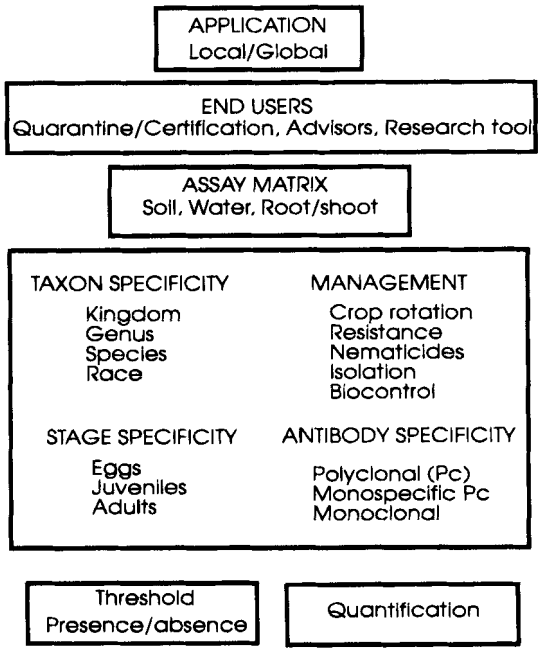

Fig. 1. Practical and theoretical considerations in the development of an immunoassay for the diagnosis and quantification of plant-parasitic nematodes (for details see text).

Taxon specificity refers to the taxonomic level of the nematode group at which diagnosis has to operate and the other organisms that will also be present in the sample to be assayed; these will be dependent on the nematode extraction procedure and the matrix from which the nematodes have to be discriminated (it should be remembered that in root/shoot assays the antibodies must not cross-react with the plant material). Stage specificity is also an important parameter which needs to be taken into account. Plant-parasitic nematodes undergo development from an egg stage, through four moults, to an adult, and, during this growth and development, there is differential expression of antigens;[12,13] this must therefore be taken into account when developing the assay. The above factors must also be assessed in the light of management strategies which can be employed and the work that is required to obtain a suitable antibody. The application of a broad-spectrum nematicide requires a less rigorous level of diagnosis than the implementation of planting a resistant cultivar which carries resistance, the latter requiring identification of a particular pathotype or race. Once all this is done, all that remains is to decide on the strategy to obtain an antibody that meets the requirements of the assay. Polyclonal antibodies tend to have high levels of sensitivity but are not usually discriminative as they are highly cross-reactive across nematode genera. Although they are relatively cheap to produce they have the disadvantage that different batches of antisera can react differently in the same assay. An intermediate approach is to use monospecific polyclonal antibodies which, if made to a diagnostic protein, can combine a high degree of discrimination with relatively high sensitivity. This latter approach obviously has the disadvantage that a diagnostic protein needs to be identified and

then purified; also, antibodies produced in this manner may contain cross-reactive epitopes. Monoclonal antibodies, which can be selected for any particular level of discrimination, are highly reproducible but tend to be less sensitive and are very expensive to produce. Before embarking on the costly and time-consuming process of making a new antibody it is well worth checking whether suitable antibodies are already available; screening monoclonal antibody libraries which have already been produced could identify suitable antibodies without the need for time-consuming immunisation schedules. The last aspect of developing a quantitative immunoassay concerns the definition of the upper and lower thresholds of the assay; a threshold assay, developed as either positive or negative above or below a certain threshold respectively, is simply a very restricted form of quantitative assay.

Root-knot nematodes and potato cyst nematodes are both sedentary endoparasites which follow very similar life-cycles. However, there are differences in these life-cycles which have important consequences for the development of an immunological assay. These two types of nematode present different problems and different challenges.

3 THE NEMATODE LIFE-CYCLES

Second-stage juveniles hatch from eggs and migrate towards the host root system, which they normally invade just behind root tips. After invasion the nematodes migrate through the root tissue (root-knot nematodes inter-cellularly and potato cyst nematodes intra-cellularly) until they identify a suitable cell which they appropriate and turn into a feeding cell. In the case of root-knot nematodes (RKN) the cell enlarges and becomes a multinucleate giant cell, whereas, for cyst nematodes, cell-wall breakdown occurs leading to the formation of a syncytium. In each case these cells become metabolically active and an understanding of the molecular mechanisms involved may lead to diagnostic markers which will relate to pathotype or race. It is at this point that the nematodes become sedentary and undergo three further moults to produce an adult female.[14] In potato cyst nematodes (PCN), which reproduce amphimictically, the developing female erupts through the root cortex and is exposed on the surface of the root; the eggs which are produced remain within the female, which eventually tans and develops into a cyst.[15] Potato cyst nematodes are monocyclic, having only one generation per year. In root-knot nematodes the root system produces a gall around the developing females and they usually remain embedded in the root.[16] The most important groups of root-knot nematodes reproduce by parthenogenesis and are polycyclic with several generations per year; the fact that RKN are polycyclic and can reproduce very rapidly means that the required threshold of detection is much lower than for PCN and the assay must be much more sensitive. Once the adult female starts to lay eggs they are extruded into an egg sac which may, or may not, be exposed on the root surface, from which they can immediately hatch. A major contrast between the two groups of nematodes is that RKN are polyphagous and can reproduce on a number of different species of crop plants, whereas PCN, as their name implies, are restricted to potatoes and a small number of other solanaceous plants.

3.1 Immuno-diagnosis of potato cyst and root-knot nematodes

The most recent developments for the identification of both PCN and RKN have involved polyacrylamide gel electrophoresis (PAGE). The differentiation of *Globodera rostochiensis* (Wollenweber) Behrens from *G. pallida* (Stone) Behrens is usually performed by homogenising cysts containing eggs and second-stage juveniles of PCN and using isoelectric focusing (IEF) techniques to identify species specific markers.[17] The identification of RKN involved the homogenisation of female RKNs and analysis by isozyme electrophoresis and staining for esterase.[7] For the initial production of antibodies, the antigens selected for the immunisations were eggs and juveniles for PCN and females for RKN, as these stages had been found previously to be useful in distinguishing species of PCN[17] and RKN.[7] The production of monoclonal antibodies (Mabs) was then done using standard techniques.[13,18] The initial screening of the antibodies produced by the hybridoma cell lines was done on protein homogenates of the species and stages of nematode that needed to be differentiated. For PCN this involved screening Mabs against *G. rostochiensis* and *G. pallida*, as the assay was designed for use locally in Europe and these were the two species that represented a problem for which there were a variety of management options available. In the case of RKN, the Mabs were screened against the three major species, the basis of which was that species differentiation was linked to the differential host range test.[19]

The use of Mabs for the immunological differentiation of the two species of PCN has been described.[18,20] The Mabs produced by Schots *et al.*[20] were not species-specific because they not only cross-reacted with the two species of PCN but also with other species of nematode; however, by combining a panel of antibodies they could differentiate the two species as each antibody had a different affinity for the antigens of the different nematodes. The two Mabs produced to PCN by Robinson *et al.*[18] did not have the problem of cross-reactivity since they only recognised species of the genus *Globodera*. Because *Globodera* species from the *S. tabacum* complex

are not present in Europe the Mabs can be considered specific to *G. rostochiensis* and *G. pallida*. It was fortuitous that further research showed that these antibodies recognised the same diagnostic markers at pI 5·7 for *G. pallida* and pI 5·9 for *G. rostochiensis* as those identified by Fleming and Marks.[17] Western blotting after SDS-PAGE showed that these two proteins, with different pIs, had the same molecular weight of 34 kD and that the antibodies were weakly cross-reactive (Fig. 2). Further immunisations with the purified diagnostic markers at pI 5·7 and pI 5·9 produced Mabs with similar levels of specificity. Subsequent characterisation of these diagnostic markers has shown that they only recognise viable eggs (Dunn, Curtis and Evans, unpublished data). For RKN three Mabs were selected which could, in an assay using females, differentiate the species by ELISA and dot blot (Fig. 3). These Mabs were highly cross-reactive in Western blot analysis and it was impossible to separate *Meloidogyne incognita* (Kofoid & White) Chitwood from *M. javanica* (Treub) Chitwood in a qualitative test.[13] Western blotting of isoelectric focused (IEF) gels and the probing of blots with each of the Mabs did not show differences in isoelectric points of the antigens between the different species of RKN (Davies and Fargette, unpublished data). In an attempt to differentiate *M. incognita* from *M. javanica*, species-specific esterase bands have been purified and Mabs produced; although it is now possible to differentiate these two species, the Mabs also cross-react with other

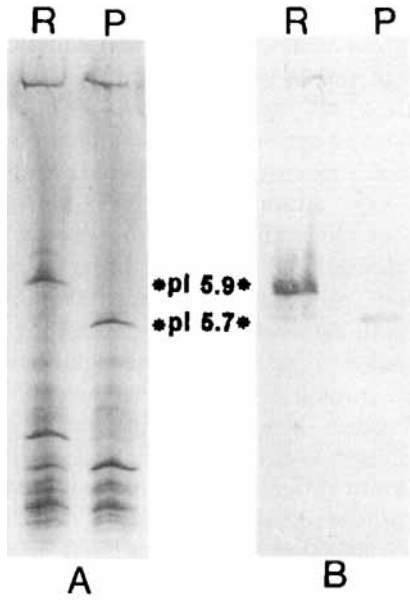

Fig. 2. Isoelectric focusing gel of cyst homogenates of *Globodera rostochiensis* and *G. pallida*, gel showing diagnostic markers at pI 5·7 and 5·9 (A) Coomassie blue staining (B) Immunoblot probed with MAC 357 (R = *G. rostochiensis*; P = *G. pallida*).

RKN (Fig. 4; Ibrahim and Davies, unpublished data). As the levels of cross-reactivity of the Mabs used to identify the species of PCN were sufficiently low as to make them irrelevant, it was possible to identify samples containing mixed populations of PCN (Dunn,

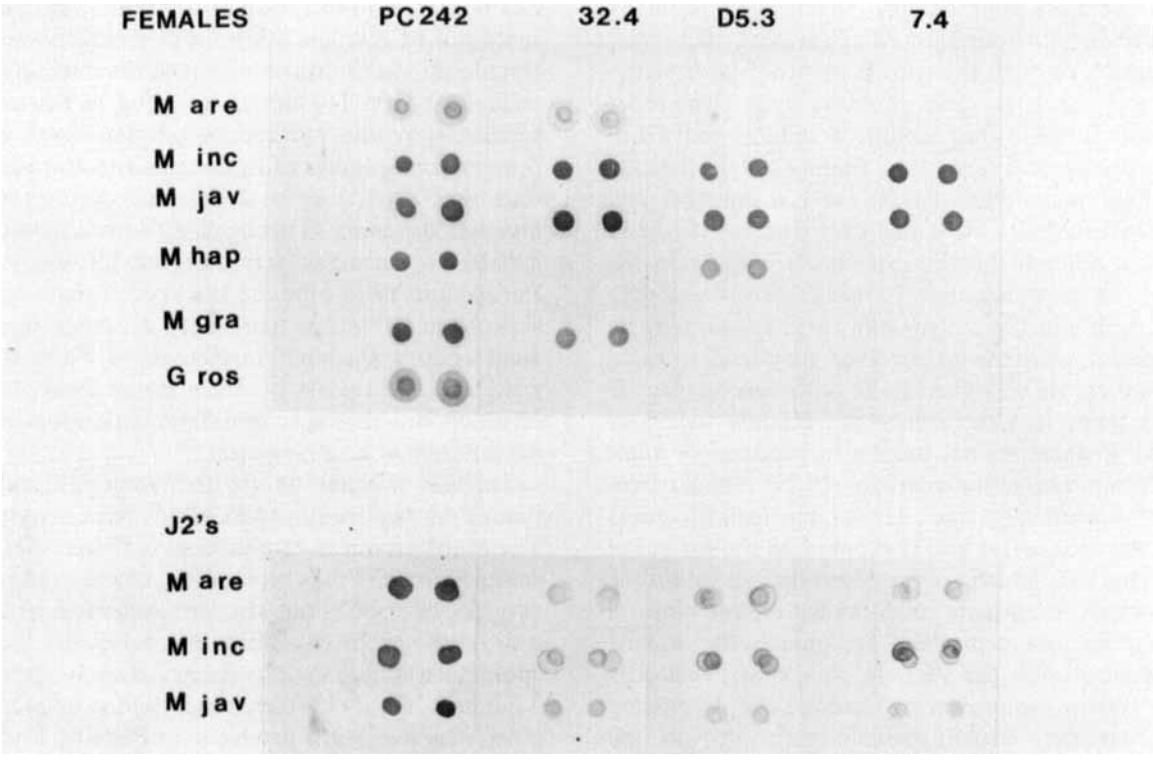

Fig. 3. Dot-blot immunoassay of females and second-stage juveniles (J2s) of a number of plant parasitic nematodes probed with polyclonal antibody PC 242 and monoclonal antibodies 32·4, D5·3 and 7·4 (M are, *Meloidogyne arenaria*; M inc, *M. incognita*; M jav, *M. javanica*; M hap, *M. hapla*; M gra, *M. graminicola*; G ros, *Globodera rostochiensis*).

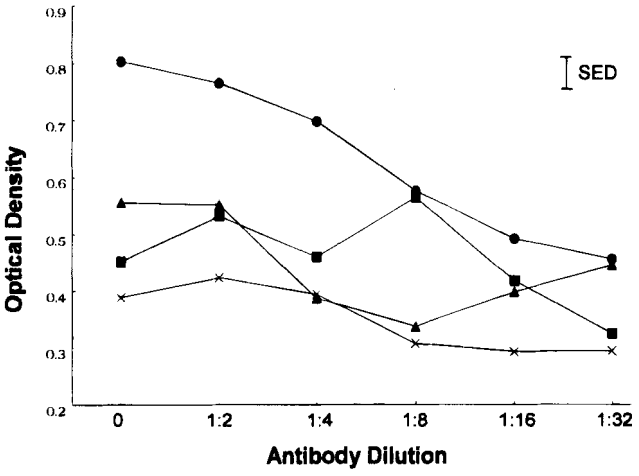

Fig. 4. ELISA showing the differential recognition of a monoclonal antibody raised to a purified esterase of (●) *Meloidogyne incognita*; (■) *M. arenaria*; (×) *M. javanica*; (▲) *M. hapla.*

Curtis and Evans, unpublished); in contrast, this was not an approach that was considered for RKN as the levels of cross-reactivity were substantially higher.

3.2 Immuno-quantification of potato cyst and root-knot nematodes

An important aspect of plant parasitic nematode management is the testing of a particular site for the presence of nematodes prior to the planting of a crop. Traditional methods of quantification rely on laborious extraction techniques together with manual counting using a microscope. Quantification could normally be carried out only at the level of genus prior to the development of electrophoretic techniques.[21] However, electrophoretic techniques are not easy to adapt for quantitative purposes. Immunoassays now offer the potential to combine identification and quantification in one single step and also have the potential for automation. Hitherto, immunoassays for diagnosis have been developed on readily identifiable stages of the nematode which have been relatively uncontaminated with other nematodes and other soil and root material. The challenge of a soil assay is to identify the desired nematode and quantify it amongst an array of other nematodes and soil fauna and flora. It is useful to consider the assay from two aspects, first identifying the desired nematode and then the release of the target antigen in a manner which is proportional to the number of nematodes present. As seen above, several antibodies have been selected as useful in diagnosis which may be useful in quantification assays.

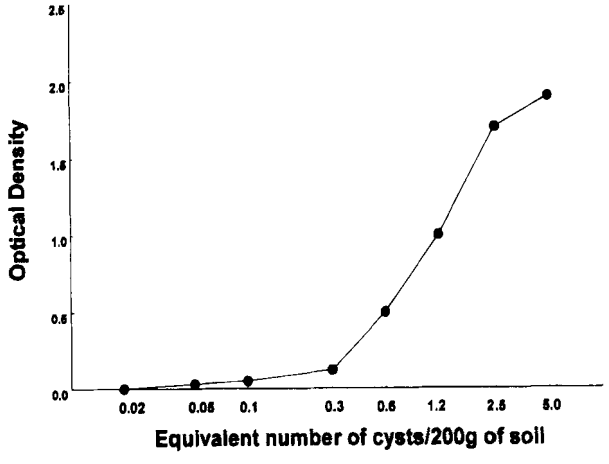

Fig. 5. Quantitative immunoassay for PCN: a Fenwick can float extract from 200 g soil containing *Globodera pallida* cysts sonicated and the supernatant tested in an indirect DAS-ELISA detected with MAC 356.

Several attempts have been made to quantify PCN and RKN nematodes directly in soil homogenates but this approach has so far proved unsuccessful and some level of nematode extraction from soil has proved necessary. It was possible to quantify second-stage juveniles of RKN with genus-specific Mabs following the extraction of the nematodes by centrifugation[22] and the subsequent use of ballotini beads as a method of antigen release. However, even though several different types of ELISA format were tested, no assay was sufficiently sensitive to quantify juveniles at the required threshold of one juvenile per gram of soil. The diagnostic antigens which had been selected were either not present in sufficient quantity or were not being released satisfactorily. The Mabs produced by Schots *et al.*[20] were used to quantify the two species of PCN from clean extracts of cysts, whilst the Mabs produced by Robinson *et al.*[18] were successful in quantifying PCN from soil extracts (Fig. 5).[23] Although the use of balottini beads was not successful in releasing antigens from either PCN cysts or eggs, both sonication and homogenisation of crude Fenwick can[24] extracts containing cysts proved that enough antigen was released to detect approximately 3×10^{-3} cysts g^{-1} soil (*c.*0·9 eggs g^{-1} soil). Therefore, the abundance and ease of release of diagnostic antigen from cysts were important factors in the development of the quantitative assay for PCN.

4 DISCUSSION AND FUTURE PROSPECTS

The research presented here shows that the development of an immunological assay to identify and quantify PCN is much more advanced than that for RKN, and it is instructive to compare and contrast the nematodes, as this brings into focus some of the important factors to be considered in the development of an assay. PCN are not indigenous to Europe and the two species present were shown to be serologically distinct even before they were identified as separate species on morphological grounds.[6] Whether or not the assay would

be useful in the area of the South American Andes, which is the centre of origin for PCN where they co-evolved with their host plants (*Solanum* spp) would seem rather doubtful.[25] PCN are genetically much more heterogeneous than RKN which, by comparison, have maintained a high degree of homogeneity through their parthenogenetic mode of reproduction[26] and this heterogeneity has greatly helped in the development of the immuno-diagnosis of the two nematode species. Another great help was that eggs and juveniles in cysts could be removed from the soil with relative ease. Added to this was the advantage that the diagnostic markers were abundant and easily released. These factors collectively facilitated the development of a successful assay.

The polyphagous life-style of RKN and their genetic homogeneity suggests that diagnosis and quantification at the level of genus is sufficient for practical purposes, as no morphological or biochemical markers which are predictive of host range have yet been identified.[6] This contrasts with PCN, where the diagnostic antigens which separate *G. pallida* from *G. rostochiensis* are useful in predicting whether a particular population of PCN in the UK will be virulent or avirulent on potatoes containing the H1 gene. This gene confers resistance to some populations of *G. rostochiensis*.[15] There is much interest in understanding the molecular mechanisms by which nematodes establish feeding cells[27] and it is likely that such studies will eventually lead to the production of Mabs and DNA probes which will be predictive of host range; these probes will be the basis of producing diagnostic tests which will be able to identify nematode pathotypes and races.

The basis of a quantitative immunoassay for PCN has now been established at the species level and an assay for RKN at the genus level is not far away. However, to date, no research has been aimed at developing assays for migratory ecto- and endo-parasitic nematodes. For such nematodes, samples are likely to contain mixed stages of the nematode and the development of an assay for this group of nematodes will present a new set of challenges.

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